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Salinity Has no Effect on Polysomatic Pattern in Seedlings of *Trifolium pratense* and *T. repens*

Valéria Kocová*, Dominika Bubanová, Albert Rákai, Vladislav Kolarčik and Pavol Mártonfi

Department of Botany, Institute of Biology and Ecology, P.J. Šafárik University, Mánesova 23, 04001 Košice, Slovakia

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Endopolyploidy is a condition of a cell containing reduplicated genetic material in its nucleus. Cells with the nuclei of different ploidy levels are often present within a single polysomatic organism. Endoreduplication is thus a modified cell cycle that omits cytokinesis and leads to chromatin replication in the endopolyploid cells. This study aimed to research the effect of salinity on endopolyploidy of *Trifolium pratense* and *T. repens*. Both species are important pasture legumes and belong to the genus *Fabaceae* with the well documented endopolyploidy occurence. Endopolyploidy levels in the seedlings treated with 0, 30, 60, 90 and 120 mM NaCl were investigated by flow cytometry. The seedling organs were evaluated during three ontogeny stages. The cytometric data plotted on a histogram showed the presence of 2C-16C nuclei in *T. pratense* and 2C-8C in *T. repens*. The hypothesis that salinity induces additional endocycles was not confirmed. Our results show that the distribution of nuclei among ploidy levels does not differ markedly between the treatment groups and the control ones. Additionally, only minor changes were observed among the endoreduplication indexes (EI) of plant organs after exposure to various salt concentrations. Endopolyploidy patterns within the salt-treated seedlings during ontogeny are similar to the controls. We suggest that endopolyploidy in *Trifolium* species is a conserved genetic trait, rather than an adaptation to salinity stress. The analyses of the roots of *T. pratense* at stage III show that with the increased concentrations of NaCl the length of roots decreased, but no evident changes in endopolyploidy occured.

Keywords: endopolyploidy, endoreduplication, *Trifolium*, salinity, stress

INTRODUCTION

Endopolyploidy is a common element of development and physiology of many Angiosperms. It is characterised by repeated multiplications of the genetic material within nuclei, which in turn do not undergo cytokinesis. Some plant groups attain a higher degree of endopolyploidization than others. Several factors are in play but taxonomic position is the major one (Barow and Meister, 2003; Bainard et al., 2012). Endopolyploidy levels are specific for some tissues and organs (Barow and Meister, 2003; Barow, 2006). The switch from mitosis to endoreduplication is irreversible and controlled by genetic, developmental and environmental signals (Maluszynska et al., 2013). Endopolyploidy was observed in the early development of plants, e.g., *Chenopodium quinoa* (Kolano et al., 2008), *Beta vulgaris* (Sliwinska and Lukaszewska, 2005) and *Trifolium pratense* (Straková et al., 2014). These works refer to endoreduplication as an essential part of normal development of seedlings of many plant species. In fact, the changes in endopolyploidy levels during ontogeny and the tissue-specific status of endopolyploidy indicate that endoreduplication is spatially and temporally regulated (Maluszynska et al., 2013).

The studies have revealed several roles of endopolyploidy, such as the control of organ growth (Sugimoto-Shirasu and Roberts, 2003; Cookson et al., 2006; Massonett et al., 2011), increasing of metabolism and utilization of the endocycle in stress-response pathways (Scholes and Paige, 2015). In endopolyploid plants, more endocycles are induced under the conditions of environmental stress, which can be provoked by abiotic and biotic factors such as light (Gendreau et al., 1998; Kudo and Mii, 2004; Kinoshita et al., 2008; Gegas et al., 2014), temperature (Engelen-Eigles et al., 2000), symbionts (Kondorosi and Kondorosi, 2004; Wildermuth, 2010), mycorrhizae (Bainard et al., 2011) and nematodes (de Almeida Engler and

* Corresponding author, email: valeria.kocova@upjs.sk

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Gheysen, 2013). Less attention has been paid to salt stress and associated water deficiency. Salt treatment induced endoreduplication in root cells of *Sorghum bicolor* or *Allium cepa* and in cali of *Nicotiana bigelovii* (Ceccarelli et al., 2006; Bennici et al., 2008). Water deficiency reduced the cell size of leaves of *Arabidopsis thaliana* and the kernel growth in maize and these were in correlation with an endopolyploidy level decrease (Cookson et al., 2006; Artlip et al., 1995). Scholes and Paige (2015) suggested that endoreduplication is employed as a plastic response to mitigate the effects of environmental stress. Plants typicaly respond to stress by increasing endopolyploidy beyond their 'normal' level.

Trifolium pratense L. and *T. repens* L. belong to the most important pasture legumes and are widely distributed in temperate regions throughout the world (Abberton, 2007). *T. pratense* is diploid (2n = 14) with 2C = 0.9 pg DNA and *T. repens* is tetraploid (2n = 32) with 2C = 2.48 pg DNA (Kocová et al., 2014). They are phylogenetically distinct: *T. pratense* belongs to the section *Trifolium* and *T. repens* to the section *Trifoliastrum* (Ellison et al., 2006). Recent studies showed that these two species are polysomatic in general; enhanced endopolyploidy levels were detected in *T. pratense* throughout its lifespan, from seeds to flower senescence (Kocová and Mártonfi, 2011; Kocová et al., 2014, Straková et al., 2014). Kocová et al. (2014) demonstrated that these two species have different endopolyploidy levels. Taxonomic affiliation seems to be the major factor impacting endopolyploidy in these species.

Salinity is a very destructive abiotic stress which limits crop productivity. It is a huge environmental problem in agricultural crop lands (Pitman and Läuchli, 2002; Munns and Gilliham, 2015; Shrivastana and Kumar, 2015). Many forage legumes are cultivated in irrigation areas with this problem. *T. pratense* has been determined as a highly salt-sensitive plant (Mandić et al., 2014). A decrease in germination and growth of seedlings in *T. pratense* was demonstrated with an increase of NaCl concentration (Asci, 2011; Mandić et al., 2014). Germination and subsequent development of seedlings are the crucial life phases of plants.

With regard to the role of endopolyploidy in early plant development under the salt stress conditions, the aim of this study was to estimate how endopolyploidy levels in individual organs of *T. pratense* and *T. repens* change during three ontogeny stages under application of various NaCl concentrations. Additionaly, we aimed to map the patterns of polysomaty in *T. repens* during the early phase of ontogeny – for the first time.

MATERIAL AND METHODS

PLANT MATERIAL

For the study we used seeds of *T. pratense* cultivar 'Nike' and *T. repens* 'Rivendel'. Twenty-five individual seeds were placed into Petri dishes with a piece of filter paper and 10 ml NaCl in the following concentrations: 0, 30, 60, 90 and 120 mM. The Petri dishes were sealed to prevent evaporation. The seeds were incubated in darkness at laboratory temperature (for 2 or 3 days). After the completion of germination (radicle protrusion), the seeds with the radicle penetrating the seed coat were transferred into plastic boxes with an openable cap and small holes at the top. 15 ml of the solutions in the concentrations mentioned above were pipetted onto filter paper supported on glass beads, onto which the seedlings were placed. The boxes were stored in a controlled environment (light 12 hours / dark 12 hours). The plants were harvested for analyses after they underwent one of the three ontogenic stages: seedlings with the cotyledons fully opened (stage I), seedlings with the first leaves developed (stage II), seedlings with the second leaves developed (stage III). To estimate the effect of salinity on the organ size and endopolyploidy level, the length of *T. pratense* roots was measured in all salt treatments (0, 30, 60, 90 and 120 mM) at stage III.

FLOW CYTOMETRY

Evaluation of endopolyploidy. Polysomaty of the organs was evaluated by flow cytometry. We prepared the samples from the following organs: root, hypocotyl, cotyledon, first leaf, petiole of the first leaf (first petiole), second leaf and petiole of the second leaf (second petiole). Every individual sample of fresh plant material was chopped in a Petri dish with a razor blade together with 1 ml GPB (General purpose buffer 0.5 mM spermin.4HCl, 30 mM sodium citrate, 20 mM MOPS, 80 mM KCl, 20 mM NaCl, 0.5% (v/v) Triton X-100 pH 7.0) prepared according to Loureiro et al. (2007). The resulting mixture was filtered through 42 μm nylon mesh filter. This suspension was supplemented with 10 $\mu g.ml^{-1}$ propidium iodide (PI) and 10 $\mu g.ml^{-1}$ RNase.

Usually 8–12 repetitions in *T. pratense* and 4–8 repetitions in *T. repens* per organ/concentration/stage were analyzed. Lower numbers of duplicates or none were prepared in the cases when the lethal effect of salt was critical for the seedlings. All flow cytometric analyses were carried out by Partec CyFlow ML (Partec Gmbh, Münster, Germany) equipped with an argon-ion laser tuned at a wavelength of 532 nm. This flow cytometer is housed at the Institute of Biological

and Ecological Sciences, P.J. Šafárik University in Košice (Slovakia). The histograms were displayed on a logarithmic scale (x-axis). The resulting data were analyzed using FloMax Software 2.7 (Partec Gmbh, Münster, Germany). The measurements were made with an effort to reach approximately the same cv (%) for all samples. The number of nuclei per individual peak was recorded on counts vs. PI fluorescence histograms. Then the percentage of nuclei of individual DNA levels in each sample was calculated and the acquired data were used to calculate the endoreduplication index (EI) (Bainard et al., 2012) according to the formula of Barow and Meister (2003). EI is averaging the number of endocycles undergone by each nucleus:

$$EI = \frac{(0 \times n_{2C} + 1 \times n_{4C} + 2 \times n_{8C} + 3 \times n_{16C}...)}{(n_{2C} + n_{4C} + n_{8C} + n_{16C}...)}$$

where n represents the number of nuclei of the corresponding ploidy level. The samples with EI less that 0.1 are not considered endopolyploid (Barow and Meister, 2003).

Determination of genome size. Genome sizes [pg] of *T. pratense* cultivar 'Nike' and *T. repens* 'Rivendel' were evaluated by flow cytometry. Preparation of the samples for genome size estimation was analogous to the protocol used for endopolyploidy measurements. The same flow cytometer was utilized. The first leaf of *T. pratense* was used and chopped together with the reference standard, *Solanum lycopersicum* cv. Stupické (2C = 1.96 pg DNA, Doležel et al., 1992). The first leaf of *T. repens* was evaluated with *Zea mays* CE-777 (2C = 5.43 pg DNA, Lysák and Doležel, 1998). Nine repetitions were carried out for both species. The histograms were displayed on a linear scale (x-axis). The coefficients of variation (CV) of the G0/G1 peaks of both our species and the internal standards did not exceed 5%. The data were analyzed with FloMax 2.7 (Partec Gmbh, Münster). DNA content was calculated according to Doležel and Bartoš (2005): Sample 2C DNA content = 2C sample peak mean / 2C standard peak mean × standard 2C DNA content [pg].

STATISTICAL METHODS

Exploratory data analysis was done in Microsoft Office Excel 2007. If the requirements of ANOVA were met, then ANOVA test (and Tukey's post hoc test for pairwise comparisons) was applied to test the mean difference of EI between treatments within specific organs. In few cases the requirements of ANOVA were not met and then Kruskal-Wallis test (with Mann-Whitney post hoc test with Bonferroni corrected p values to determine significance) was applied instead. These tests were performed in Past 3.14 (a significance level of 0.05 was used). Graphs were created in ggplot2 package (Wickham 2009) in R (ver. 3.2.4) environment (R Core Team, 2016).

RESULTS

The genome size of *T. pratense* cultivar 'Nike' was estimated as 0.91 ± 0.018 pg (mean ± SD) and of *T. repens* 'Rivendel' as 2.22 ± 0.022 pg.

The highest concentration of NaCl showed vast negative effects on *Trifolium* species; therefore, the results for the roots at stage III of *T. pratense* treated with the 120 mM solution are not present here (Table 1). The seedlings of *T. repens* showed higher sensitivity to salinity than the seedlings of *T. pratense*. The results for *T. repens* at stage I include the data from 90 mM and 120 mM NaCl treatment, whereas the data for these concentrations at stage II and stage III are missing, since the plants were not able to grow in these NaCl concentrations (Table 2).

We studied the relationship between the organ size and the DNA content. The differences in the root length between the control and the salt treatments were obvious at stage III. The average lenght of *T. pratense* roots for 0 mM NaCl was 7.65 ± 2.51 cm (mean ± SD), for 30 mM 9.34 ± 3.02 cm, for 60 mM 6.62 ± 2.9 cm, for 90 mM 2.36 ± 1.55 cm and for 120 mM 0.84 ± 0.35 cm.

Developing organs of *T. pratense* contain cells with the nuclei of 2C, 4C, 8C and even 16C in some cases (Fig. 1). *T. repens* shows very low amounts of the 8C nuclei and the predominant 2C and 4C nuclei are present (Fig. 2). The seedlings of *T. pratense* and *T. repens* are polysomatic during the early stages of their ontogeny in both the control and the treatment groups. Exceptions were found for the first and the second leaves of *T. pratense* and the first and the second leaves and petioles of *T. repens*. These organs are considered non-endopolyploid (EI was under 0.1, Barow and Meister, 2003) (Tables 1, 2).

The effect of salinity on ploidy levels and the EI of the cells in developing organs can be discussed in two aspects. Firstly, we have to consider the effect of various concentrations of salt solution applied on individual organs compared to the control group. The second aspect deals with the long-term effect of salt concentration on EI of organs within the salt treatments during their ontogeny.

First aspect. In general, salt concentrations did not induce more endocycles in comparison to the control and only moderate changes in the percentage of nuclei were detected in salt-treated plants of *Trifolium* species.

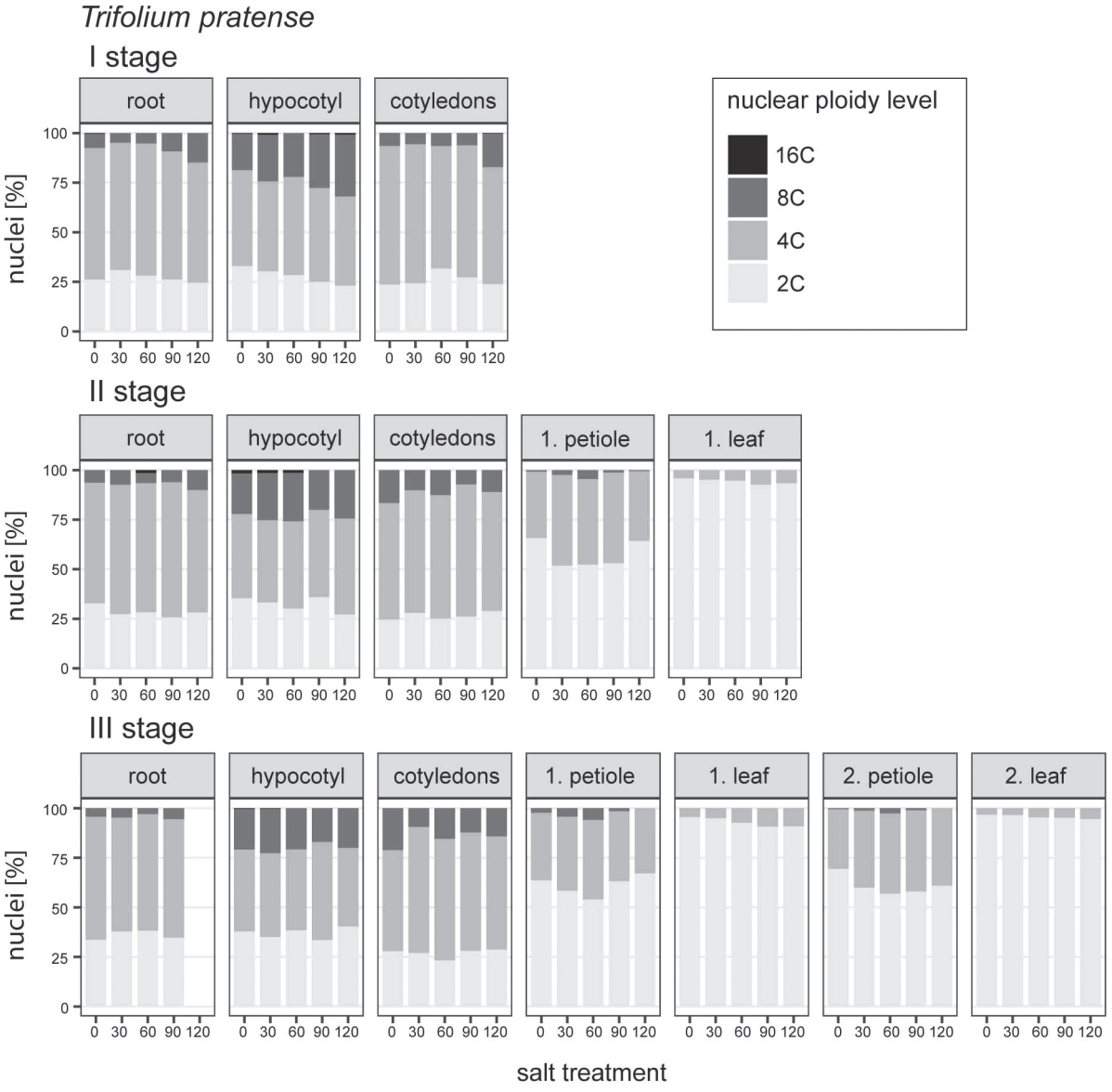

Fig. 1. Percentage of nuclei in organs of *T. pratense* seedlings within different NaCl concentrations [0–120 mM] during three ontogenetic stages.

Quantitatively significant differences for *T. pratense* were only found in the roots, the hypocotyls and the cotyledons at stage I, the first petioles and the first leaves at stage II and the first petiole at stage III (Table 1). The most significant difference in EI was found at stage I between the hypocotyls of 120 mM (1.1) and 0 mM NaCl (0.86). In general, we observed only moderate increase in EI of the salt-treated roots (at stage II), hypocotyls (at stages I and II), first and second petioles, first and second leaves, compared to the control (Table 1). EI of the hypocotyls at stage III rose only with the application of 30 mM salt solution. The cotyledons of the experimental plants in general (except for the 120 mM NaCl solution treatment at stage I) show lower EI values than those of the control group. Inspecting the first and the second petioles, we found EI slightly rising as a response to the salt treatment. EI of the leaves also increased, altough it still could not formally be considered endopolyploid. Similarly, EI of the first and the second leaves of *T. repens* increased with higher salt concentration, but always remained under 0.1. EI of the first petiole at stage II treated with 60 mM salt solution increased above 0.1 with significant difference in comparison to

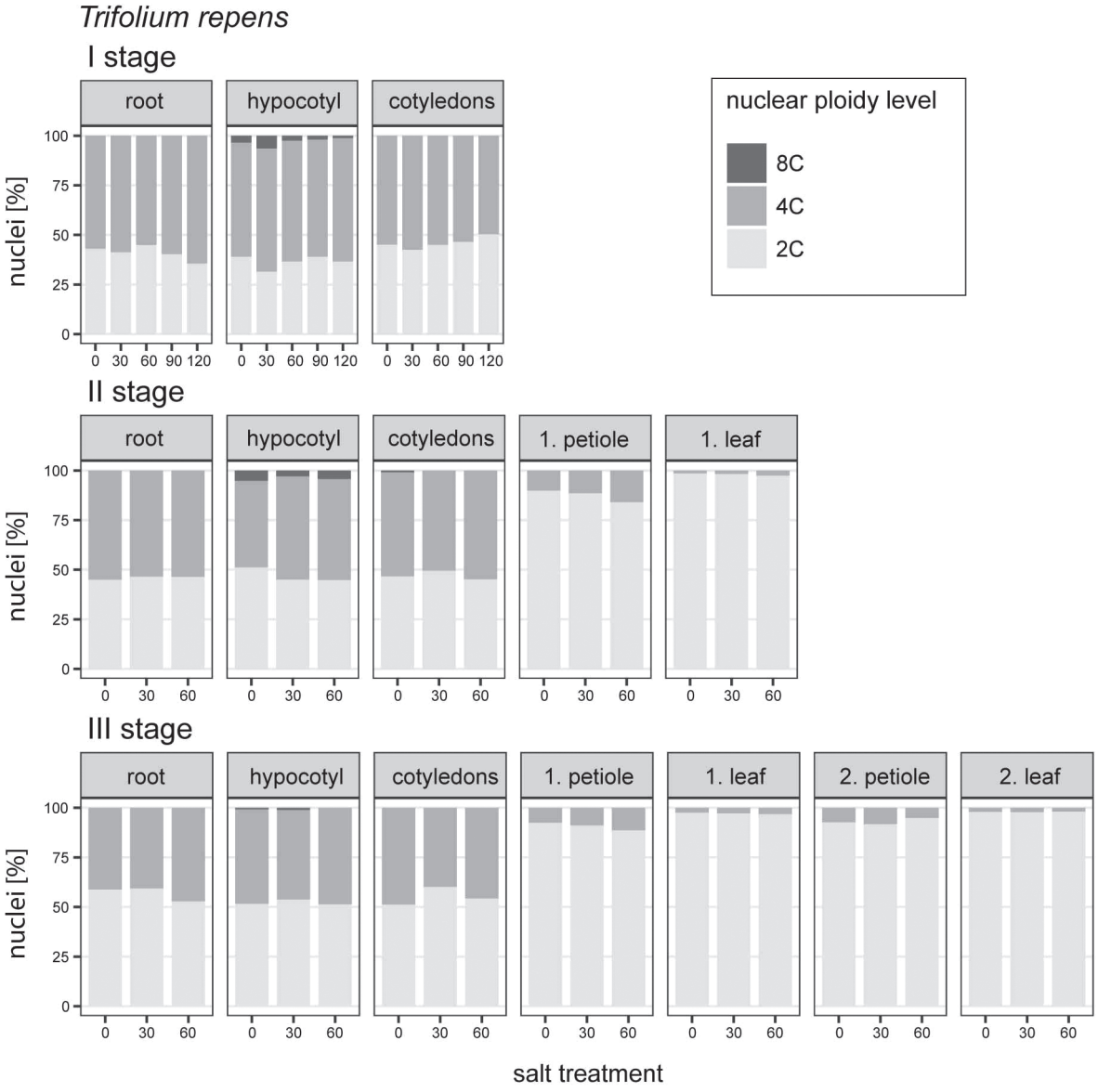

Fig. 2. Percentage of nuclei in organs of *T. repens* seedlings within different NaCl concentrations [0–120 mM] during three ontogenetic stages.

the control (ANOVA, $p < 0.05$; Table 2). Other organs of *T. repens* showed the salt-induced endopolyploidy patterns similar to *T. pratense* (Suppl. Fig. S1).

Second aspect. EI for both *T. pratense* and *T. repens* roots, hypocotyls and first petioles was predominantly decreasing during the ontogeny stages (Tables 1, 2). An opposite pattern was detected for the first leaves. A single difference between the species was detected: while endopolyploidy of the cotyledons in *T. pratense* increased with ontogeny, it decreased in *T. repens* (Tables 1, 2; Suppl. Fig. S1).

In general, the tendencies of EI are similar in *T. pratense* and *T. repens*, irrespective of the salt concentration or the ontogeny stage (Suppl. Fig. S1).

DISCUSSION

T. pratense is among the well known polysomatic species (Barow and Meister, 2003; Kocová et al., 2014; Straková et al., 2014). Here we document and prove, along with the previous study (Kocová et al., 2014), that *T. repens* is polysomatic at early

TABLE 1. EI of organs of *T. pratense* seedlings within different NaCl concentrations [0–120 mM] during three ontogenetic stages [I–III].

	I	II	III
		root	
0 mM	0.816 ± 0.056 a	0.735 ± 0.080 ns	0.706 ± 0.053 ns
30 mM	0.739 ± 0.085 a	0.799 ± 0.071	0.668 ± 0.076
60 mM	0.777 ± 0.040 a	0.796 ± 0.103	0.646 ± 0.125
90 mM	0.829 ± 0.099 ab	0.803 ± 0.065	0.708 ± 0.182
120 mM	0.953 ± 0.176 b	0.818 ± 0.145	–
		hypocotyl	
0 mM	0.860 ± 0.050 a	0.883 ± 0.127 ns	0.832 ± 0.140 ns
30 mM	0.947 ± 0.116 ab	0.933 ± 0.085	0.879 ± 0.110
60 mM	0.937 ± 0.124 ab	0.969 ± 0.099	0.823 ± 0.140
90 mM	1.030 ± 0.141 ab	0.840 ± 0.063	0.835 ± 0.097
120 mM	1.096 ± 0.132 b	0.972 ± 0.150	0.796 ± 0.172
		cotyledons	
0 mM	0.828 ± 0.080 ab	0.919 ± 0.125 ns	0.932 ± 0.133 ns
30 mM	0.812 ± 0.063 ab	0.821 ± 0.074	0.824 ± 0.083
60 mM	0.748 ± 0.088 a	0.874 ± 0.086	0.922 ± 0.070
90 mM	0.788 ± 0.056 a	0.810 ± 0.043	0.841 ± 0.111
120 mM	0.899 ± 0.080 b	0.820 ± 0.098	0.854 ± 0.073
		1. petiole	
0 mM		0.357 ± 0.106 a	0.386 ± 0.092 ab
30 mM		0.506 ± 0.076 b	0.458 ± 0.096 ab
60 mM		0.521 ± 0.072 b	0.518 ± 0.076 a
90 mM		0.481 ± 0.098 b	0.382 ± 0.110 ab
120 mM		0.362 ± 0.091 a	0.328 ± 0.035 b
		1. leaf	
0 mM		0.042 ± 0.006 a	0.045 ± 0.020 ns
30 mM		0.049 ± 0.014 ab	0.050 ± 0.027
60 mM		0.053 ± 0.015 ab	0.073 ± 0.019
90 mM		0.073 ± 0.019 b	0.093 ± 0.049
120 mM		0.066 ± 0.025 ab	0.091 ± 0.028
		2. petiole	
0 mM			0.311 ± 0.116 ns
30 mM			0.412 ± 0.088
60 mM			0.457 ± 0.108

TABLE 1. continued

	I	II	III
90 mM			0.430 ± 0.104
120 mM			0.390 ± 0.048
		2. leaf	
0 mM			0.032 ± 0.006 ns
30 mM			0.034 ± 0.008
60 mM			0.046 ± 0.018
90 mM			0.048 ± 0.017
120 mM			0.054 ± 0.023

Values are mean of EI ± standard deviation, different letters indicate homogeneous groups revealed by Tukey's or Mann–Whitney pairwise post hoc tests, if ANOVA and Kruskal-Wallis test have indicated significant differences in EI between NaCl concentrations. ns = no significant difference

and late development stages, but it does not reach the same endopolyploidy level as *T. pratense*. It is clear that polysomaty is a permanent trait throughout the whole life cycle of this crop species.

The level of endopolyploidy present in individual organs of seedlings is important with regards to their differentiation and development (Scholes and Paige, 2015). Scholes and Paige (2015) suggest that plants use endoreduplication as an adaptive mechanism to mitigate the effects of stress. Based on Scholes and Paige (2015) and with the insight into the impact of salinity on endoreduplication by Bennici et al. (2008) and Ceccarelli et al. (2006), we expected to find considerable changes in endopolyploidy levels in *Trifolium* seedlings.

The study of Ceccarelli et al. (2006) showed that an increased presence of salt noticeably enhanced endoreduplication in roots of *Sorghum bicolor*. Specifically, root hairs showed an increase in the amount of 8C nuclei and additional 16C and 32C nuclei were present. However, the proportions of the nuclei in the leaves and the vascular cylinder of *Sorghum bicolor* were similar to the controls (Ceccarelli et al., 2006). In the salt-treated root differentiation zone of *Allium cepa*, an increase of 4C and aditional 8C and 16C nuclei were observed (Bennici et al., 2008). With an increased NaCl concentration, higher DNA contents were recorded in *Nicotiana bigelovii* (Bennici et al., 2008). However, our results suggest that increased salinity does not affect endoreduplication in *Trifolium* organs of seedlings as extensively as expected. Both the control and the salt-treated plants of *Trifolium* species showed the same ploidy level distribution, meaning that the heightened salinity did not induce aditional endocycles. Aditionally, only minor differences in the distribution of nuclei among ploidy levels induced by the salt treatment were observed (Fig. 1, 2). These results show that excessive salinity does not cause significant changes in the ploidy distribution within the developing *Trifolium* seedlings (Fig. 1, 2).

We found slight (statistically insignificant) increases in EI for some organs caused by the application of salt solution to the growth medium (Tables 1, 2). This may serve the young seedlings as a means of preservation of the integrity and the function of plant organs. Consulting our results, we propose that endopolyploidy of *Trifolium* species is more likely conditioned by genetic prerequisites while less susceptible to the stress factor. Referring to the study of Ceccarelli et al. (2006), the salt-induced endoreduplication occured in the salt-adapted *Sorghum bicolor* cv. 610, while remaining unchanged in another genotype of *S. bicolor* (Dk 34-Alabama), incompetent for salt adaptation. The studies of Bennici et al. (2008) and Ceccarelli et al. (2006) together with our results suggest that the plasticity of endoreduplication is a genomic response to high salinity. It also exhibits tissue and species specificity and variation related to genetic characteristics of the studied plant.

In general, the patterns of endopolyploidy during the ontogeny stages within the organs of *T. pratense* and *T. repens* treated with salt are similar to the control group and correspond with the results of Straková et al. (2014). Again, this suggests the low impact of salinity on endopolyploidy of both species. However, ambiguous values of EI were obtained from the analyses of cotyledons of *T. pratense* (Table 1). The average EI of the cotyledons treated with 60 mM salt solution rose up from 0.75 at stage I to 0.92

TABLE 2. EI of organs of *T. repens* seedlings within different NaCl concentrations [0–120 mM] during three ontogenetic stages [I–III].

	I	II	III
		root	
0 mM	0.569 ± 0.083 ns	0.551 ± 0.051 ns	0.412 ± 0.076 ns
30 mM	0.587 ± 0.049	0.534 ± 0.082	0.407 ± 0.040
60 mM	0.550 ± 0.067	0.535 ± 0.074	0.471 ± 0.104
90 mM	0.597 ± 0.057	–	–
120 mM	0.644 ± 0.025	–	–
		hypocotyl	
0 mM	0.644 ± 0.078 ns	0.540 ± 0.070 ns	0.492 ± 0.114 ns
30 mM	0.749 ± 0.104	0.579 ± 0.070	0.473 ± 0.100
60 mM	0.658 ± 0.095	0.595 ± 0.096	0.486 ± 0.050
90 mM	0.628 ± 0.081	–	–
120 mM	0.645 ± 0.102	–	–
		cotyledons	
0 mM	0.549 ± 0.075 ns	0.542 ± 0.040 ns	0.487 ± 0.042 ns
30 mM	0.575 ± 0.112	0.504 ± 0.109	0.400 ± 0.095
60 mM	0.550 ± 0.057	0.548 ± 0.082	0.457 ± 0.031
90 mM	0.535 ± 0.209	–	–
120 mM	0.496 ± 0.161	–	–
		1. petiole	
0 mM		0.101 ± 0.023 a	0.075 ± 0.017 ns
30 mM		0.114 ± 0.043 a	0.088 ± 0.037
60 mM		0.160 ± 0.042 b	0.113 ± 0.040
90 mM		–	–
120 mM		–	–
		1. leaf	
0 mM		0.014 ± 0.011 ns	0.024 ± 0.019 ns
30 mM		0.017 ±0.008	0.028 ± 0.027
60 mM		0.025 ± 0.009	0.032 ± 0.021
90 mM		–	–
120 mM		–	–
		2. petiole	
0 mM			0.073 ± 0.058 ns
30 mM			0.082 ± 0.025

TABLE 2. continued

	I	II	III
60 mM			0.051 ± 0.048
90 mM			–
120 mM			–
		2. leaf	
0 mM			0.020 ± 0.010 ns
30 mM			0.022 ± 0.023
60 mM			0.019 ± 0.013
90 mM			–
120 mM			–

Values are mean of EI ± standard deviation, different letters indicate homogeneous groups revealed by Tukey's or Mann–Whitney pairwise post hoc tests, if ANOVA and Kruskal–Wallis test have indicated significant differences in EI between NaCl concentrations. ns = no significant difference

at stage III. Very high EI of the cotyledons is most likely caused by a strong metabolic activity of these organs (Maluszynska et al., 2013) further utilized for primary nutrition processes during the salinity stress.

According to several studies, cell size is positively correlated to endopolyploidy (Barow et al., 2006; Sugimoto-Shirasu and Roberts, 2003). It has been speculated that cell division, cell expansion and endoreduplication work on organ growth in conjunction with each other. Endoreduplication can aid organ development through cell expansion. Massonnet et al. (2011) describe the crucial role of endoreduplication in the leaf growth. Another study shows how shading and water deficit lead to a reduced leaf volume, lower cell numbers and endopolyploidy (Cookson et al., 2006). We elaborated on this hypothesis and found out that the lenght of roots at stage III exposed to salt varied greatly: 30 mM treated roots were longer than the control; in all other instances the lenght of the roots decreased with the treatment, from 30 mM to 120 mM. When considering the impact of various treatments on ploidy levels present in the roots, our results show the same 2C, 4C and 8C nuclei proportions as in the control group. It suggests that salt stress affects the growth of roots negatively, while at the same time the proportions of 2C, 4C and 8C nuclei are maintained. It means that the differences in the root size are apparently caused by an overall lower growth rate (the decrease in cell division rate) as the salt treatment intensifies, rather than a physiological effect of endopolyploidy changes. De Veylder et al. (2011) describe how a plant with an elevated percentage of higher-ploidy cells can use the growth potential of these endopolyploid cells to compensate for the decreased cell number caused by external factors. However, it is not our case. It seems that the roots of *T. pratense* prefer to maintain constant endopolyploidy levels and the effect of salt treatment is directly translated into the hindered tissue development and the inhibited elongation of roots without any compensation on the plant´s part.

CONCLUSION

Mandić et al. (2014) determined that *T. pratense* is a highly salt-sensitive plant, especially during germination and early seedling growth stages. Our results dealing with *T. pratense* and *T. repens* show that *Trifolium* species may not be salt-competent, because the plants conserve default ploidy levels in their organs despite the salt stress. This proves that endopolyploidy in *T. pratense* and *T. repens* is genetically fixed. Some of the detected changes in EI could stand for an attempted adaptation to salt, since maintaining of the function of the studied organs is crucially important. To sum up, salt stress may induce endopolyploidy changes in *Trifolium* species, but only to a negligible extent. We suggest that in the case of the studied species, the genetic predisposition for a certain polysomatic pattern outweighs its induction by stress.

AUTHORS' CONTRIBUTIONS

VKoc conducted and coordinated the study. VKoc carried out flow cytometry analyses. DB participated in the experimental work. VKol analyzed the data statistically. VKoc and AR wrote the manuscript. PM conceptualized the study and edited the manuscript.

All authors have read and approved the final manuscript. The authors declare no conflict of interest.

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